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# Use of Two-Dimensional Polyacrylamide Gel Electrophoresis to Identify and Classify *Rhizobium* Strains

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Fifty-seven strains of various *Rhizobium* species were analyzed by two-dimensional gel electrophoresis. Since the protein pattern on such gels is a reflection of the genetic background of the tested strains, similarities in pattern allowed us to estimate the relatedness between these strains. All group II rhizobia (slow growing) were closely related and were very distinct from group I rhizobia (fast growing). *Rhizobium meliloti* strains formed a distinct group. The collection of *R. leguminosarum* and *R. trifolii* strains together formed another distinct group. Although there were some similarities within the *R. phaseoli*, sesbania rhizobia, and lotus rhizobia, the members within these seemed much more diverse than the members of the above groups. The technique also is useful to determine whether two unknown strains are identical.

Two problems which have plagued research on the *Rhizobium*-legume symbiosis are: (i) the vagaries of *Rhizobium* species identification which show up as a difficulty both in taxonomy and in attempts to understand the nature of host-symbiont specificity, and (ii) the difficulties of recognizing a given *Rhizobium* strain, especially after it has entered a soil containing indigenous uncharacterized strains. Current species identification relies mainly on the host plant that is nodulated (9). Defined species and typical legume hosts are *R. leguminosarum* (pea), *R. phaseoli* (bean), *R. trifolii* (clover), *R. meliloti* (alfalfa), *R. japonicum* (soybean), and *R. lupini* (lupine). These species are divided into two groups, defined by the rate of growth on yeast extract media. Group I consists of the rapid-growing species: *R. leguminosarum, R. phaseoli, R. trifolii,* and *R. meliloti.* The slow-growing species are in group II, consisting of *R. japonicum* and *R. lupini.*

Nodulation specificity is not a suitable taxonomic criterion, however, because there are many examples of one strain nodulating more than one group of plants (reviewed in reference 24). For example, a strain isolated from pea nodules is named *R. leguminosarum,* but is later found to nodulate clover as well. If that strain had been isolated initially from clover, it would have been named *R. trifolii.* Furthermore, certain slow-growing strains (cowpea rhizobia) are very cross-invasive. Isolates from lotus (lotus rhizobia) include both fast-growing and slow-growing strains. Finally, relative deoxyribonucleic acid homology shows quite great variation within a given named species (3, 5).

Identifying strains of a given species in field studies is important for understanding (and perhaps manipulating) the ecology and competitiveness of *Rhizobium.* Current methods being used in strain identification generally rely on surface properties such as binding by plant lectins (2), phage typing (12), and serology (6, 19). These tests are not specific—more than one different strain can be typed identically (D. Noel, unpublished data). The tests are all vulnerable to single mutational events which alter the cell surface (i.e., "rough" mutations), and alterations in these "identifying" properties can be selected for inadvertently in nature (e.g., phage resistance).

An alternative method for strain identification has been the use of antibiotic resistance markers. This suffers from three major objections: (i) the presence of the resistance characteristic might perturb (20) the very experiment being performed (e.g., alteration in growth rate or cell permeability), (ii) there is a high incidence of antibiotic resistance already present in many *Rhizobium* strains (17), and (iii) such antibiotic resistances can be transferred between strains in the soil as well as in a nodule (7, 17).

The technique (10) using intrinsic levels of antibiotic resistance of *Rhizobium* strains overcomes only the first of these objections. Further, the appearance of numerous spurious differences in antibiotic resistance patterns between strains differing only by an auxotrophic marker (or, more properly, by a single mutagenesis) argues that the technique is inappropriate for reliable

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strain identification. In practice, this might mean that an apparently insignificant alteration, possibly selected for by experimental conditions, would render a strain unrecognizable by that method.

Clearly, a technique is needed which would: (i) allow taxonomy to be based on the overall genetic background of the cell rather than a few selected properties, (ii) be useful for estimating relatedness of very similar strains as well as very different ones, (iii) be sensitive enough to differentiate independently isolated strains that are "identical" by traditional tests, and (iv) be convenient enough for rapid and reproducible analyses. This paper describes the use of two-dimensional polyacrylamide gel electrophoresis to fill these needs and gives a preliminary organization of a number of *Rhizobium* strains based on gel patterns.

## MATERIALS AND METHODS

**Media.** Strains were grown on either RM (a minimal medium described in reference 1) or on AMA, which contains, in 1 liter: 10 g of mannitol, 1 g of yeast extract, 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.2 g of NaCl, 0.5 g of $K_2HPO_4$, 15 g of agar, and 1 ml of Fe solution. In 100 ml, the Fe solution contained: 0.67 g of $FeCl_3 \cdot 6H_2O$ and 0.42 ml of concentrated HCl.

**Bacterial strains.** All of the strains (Table 1) used in this study were obtained from J. Burton of the Nitragin Co., Milwaukee, Wis. Upon receipt of the cultures, each strain was streaked onto yeast extract-mannitol (AMA) plates and incubated at 30°C. With many of the strains, two or more colony types were observed on the plates. The predominant colony type had a smooth appearance typical of *Rhizobium*, whereas the others were smaller and rougher. Single colonies were picked from each colony type that appeared on a plate and were restreaked for purity. Single colonies were picked again and stocked. Twenty-five-milliliter cultures of each isolate were grown in yeast extract-mannitol broth to midexponential phase and then pelleted by centrifugation. The pellets were washed by suspending them in phosphate-buffered saline (50 mM $KH_2PO_4$, 50 mM $K_2HPO_4$, 150 mM NaCl). The cells were again pelleted by centrifugation, frozen, and stored at −20°C.

**Chemicals and isotopes.** Acrylamide, bisacrylamide, sodium dodecyl sulfate, and Coomassie brilliant blue R-250 were obtained from Bio-Rad Inc., Richmond, Calif. Ultrapure urea was purchased from Schwarz/Mann Co., Orangeburg, N.Y., and the carrier

TABLE 1. *Rhizobium strains and their sources*

| Strain | Parent host | Geographical source | Strain | Parent host | Geographical source |
|---|---|---|---|---|---|
| *R. meliloti* | | | 127K26 | *P. vulgaris* | Illinois |
| 102F28 | *Medicago sativa* | ? | 127K44 | *P. vulgaris* | Minnesota |
| 102F51 | *Medicago* sp. | Uruguay | 127K55 | *P. vulgaris* | Idaho |
| 102F65 | *Medicago sativa* | Prosser, Wash. | 127K49 | *P. vulgaris* | Idaho |
| 104A12 | *Melilotus alba* | California | 127K17 | *P. vulgaris* | Wisconsin |
| 104B6 | *Melilotus indica* | Arizona | *R. lupini* | | |
| 102F26 | ? | Illinois | 96A6 | *Lupinus albus* | Alabama |
| 102F45 | *Medicago sativa* | Brazil | 96B8 | *L. angustifolius* | Florida |
| 102F34 | ? | ? | 96B11 | *L. angustifolius* | Florida |
| 104A14 | *Melilotus alba* | California | 96E4 | *L. luteus* | Florida |
| *R. leguminosarum* | | | 96E8 | *L. luteus* | Florida |
| | | | 96G1 | *L. polyphyllus* | ? |
| 128C53 | *Pisum sativum* | ? | *R. japonicum* | | |
| 128C56 | *P. sativum* | ? | 61A84 | *Glycine max* | Louisiana |
| 128C82 | *P. sativum* | Minnesota | 61A88 | *G. max* | Taiwan |
| 175G9 | *Vicia villosa* | Minnesota | 61A92 | *G. max* | ? |
| 175F3 | *V. faba* | Manitoba, Canada | 61A103 | *G. max* | Indiana |
| | | | 61A119 | *G. max* | Wisconsin |
| 175P1 | *V. dasycarpa* | Wisconsin | 61A76 | *G. max* | Mississippi |
| *R. trifolii* | | | | | |
| 162E8 | *Trifolium fragiferum* | California | Cowpea rhizobium | | |
| 162T2 | *T. semipilosum* | Kenya, Africa | 176A17 | *Vigna unguiculata* | ? |
| 162Z1 | *Trifolium* sp. | Australia | 176A27 | *V. unguiculata* | Nigeria |
| 162P17 | *T. pratense* | Wisconsin | 176A32 | *V. unguiculata* | Nigeria |
| 162S31 | *T. repens* | New Zealand | 176A34 | *V. unguiculata* | Nigeria |
| 162X47 | *T. subterraneum* | California | 176A37 | *V. unguiculata* | Nigeria |
| Sesbania rhizobium | | | 176A40 | *V. unguiculata* | Brazil |
| 145A1 | *Sesbania macrocarpus* | ? | Lotus rhizobium | | |
| 145A4 | *S. macrocarpus* | Mississippi | | | |
| 145A6 | *S. macrocarpus* | Mississippi | 95C6 | *Lotus corniculatus* | Wisconsin |
| 145A10 | *S. macrocarpus* | Mexico | 95C8 | *L. corniculatus* | New York |
| 145B1 | *S. longifolia* | Alabama | 95C11 | *L. corniculatus* | Wisconsin |
| 145Z1 | *S. longifolia* | Texas | 95E6 | *L. pedunculatus* | Florida |
| *R. phaseoli* | | | 95E8 | *L. pendunculatus* | Australia |
| 127K14 | *Phaseolus vulgaris* | Wisconsin | 95G2 | *L. salsuginosus* | California |

ampholytes for isoelectric focusing were purchased from LKB Instruments, Inc., Rockville, Md.

**Two-dimensional polyacrylamide gel electrophoresis.** Cell pellets were resuspended in 0.25 ml of 10 mM tris(hydroxymethyl)aminomethane-hydrochloride (pH 7.4), 5 mM $MgCl_2$, 0.1 mg of pancreatic ribonuclease per ml, and 0.5 mg of pancreatic deoxyribonuclease per ml. The suspension was sonicated at 4°C for 30 s with the microtip probe of a Branson Sonifier W350 (Branson Sonic Power Co., Stamford, Conn.) at 40-W output. After 5 min of incubation at 4 to 10°C, the samples were frozen. Two hundred milligrams of urea and 0.1 ml of lysis buffer (16) were added, and the suspension was freeze-thawed for four cycles. The samples were centrifuged at 3,000 × *g* for 5 min, and 0.02 to 0.10 ml of the supernatant solution was loaded on the first-dimension gel. The technique used in performing two-dimensional gel electrophoresis has been described previously (16) with modifications (18). The acrylamide concentration in the second dimension was 12%.

## RESULTS

**General considerations.** Figure 1 shows the protein patterns of typical group I (g through o) and group II (a through f) rhizobia. Lines have been placed on all gels in order to provide a reference and to direct attention to regions of the gels which are diagnostic for the various rhizobial groups. At the gross level, several points are clear. (i) The distribution of proteins in the two groups is very different. The bulk of visible proteins in group I strains are acidic, whereas group II strains have their most abundant proteins at a much more basic position. (ii) Whereas the group II patterns are rather similar among themselves, the group I patterns tend to be very dissimilar overall, with similarities apparent only within small sets of strains. (iii) No obvious similarities exist between the group I and group II strains.

To examine the gel pattern relationships among 57 strains, the samples were initially run and assorted into groupings of similar patterns by two people independently in a double-blind test. The groupings by each person were essentially the same as those shown in Fig. 2 and 3 and were similar to each other despite different techniques of pattern recognition: one person scored the gels for the presence of certain multispot patterns, and the other scored the relative positions of a number of distinctive spots. It is important to note that the two methods (and, by implication, any sort of pattern recognition based on a number of spots) gave similar results. This is due to the redundancy of taxonomic information: any two strains derived (evolutionarily or by selection) from a third will share many pattern similarities with each other and even more similarities with a strain from which they were derived. As explained below, the diversity of the sesbania rhizobia and *R. phaseoli* patterns prevented both examiners from a meaningful grouping of these strains except to note their nonidentity with other groupings.

**Group II strains.** When the major diagnostic spots of the group II strains were examined, neither cowpea rhizobia nor *R. japonicum* strains could be assorted into species-specific groups (based on host plants). On the contrary, many of these strains were more closely related to lotus rhizobia or *R. lupini* than to other strains of their own inoculation group. Although it is possible that diagnostic spots for *R. japonicum* and cowpea rhizobia inoculation groups were present on the gels, such spots were not identified. In the case of both lotus rhizobia and *R. lupini,* clusters of spots diagnostic for these groups were observed and are circled on gels e and f of Fig. 1.

**Group I strains.** Although there were some striking pattern similarities within subgroups of these fast-growing rhizobia, they were much more diverse as a group than the group II strains. In some cases, the diversity was so great that the strains currently assigned to a single species seemed to be quite distantly related.

The *R. meliloti* strains formed a reasonably homogeneous group (l and n of Fig. 1), with the cluster of spots to the right of the arrow among the more easily scored of the similarities. One sesbania rhizobium (145A1) had certain similarities (Fig. 1m) in the *R. meliloti* diagnostic region just mentioned, but clear differences did exist (see especially circled regions in l, m, and n of Fig. 1). This suggested, however, that this sesbania rhizobium might possess certain *R. meliloti* determinants, and it is interesting that although this sesbania rhizobium failed to nodulate alfalfa, it was agglutinated by alfalfa lectin which was otherwise specific for *R. meliloti* strains (A. Paau, personal communication).

The patterns of all *R. leguminosarum* and *R. trifolii* strains tested (for example, see Fig. 1) were rather similar, and there were no obvious spots which could be used to differentiate members of the two inoculation groups from each other. The strains of these two inoculation groups showed a relatedness comparable to that seen with the *R. meliloti* strains, and therefore their designation as two separate species is probably inappropriate.

Lotus rhizobia (the fast-growing isolates) are rather diverse, but all representatives shared the pattern boxed in Fig. 1g. This pattern seems unique among group I strains.

Although an occasional *R. phaseoli* and sesbania rhizobium showed similarities with other strains of the same inoculation group, there were no obvious determinants which were found useful in making any meaningful grouping of strains. Assignment of either group of these strains to a single "species" is clearly too broad to be useful.

**Other considerations.** Different colony morphologies do not necessarily indicate contamination. Twenty-two (of 57) strains evidenced more than one colony type upon single-colony isolation. In all such cases, gel analysis revealed the extreme similarity of the multiple, morphologically different isolates. In general, such strains differed significantly in the intensity of one or two major spots, the remainder of the pattern being identical (see Fig. 1i, gummy, and 1k, nongummy). Such multiple forms, therefore, are not contaminants but presumably are derived from one another by a single mutational event during storage.

**Effect of growth rate.** Several rhizobium strains (162P17, 162S31, 61A76) were prepared for gel analysis by growth on minimal (RM) and rich (AMA) media. The results indicated that although differences in spot intensity did occur depending on the media, these differences were not so dramatic as to change grouping assignments. The pattern alterations induced by different media were not dramatic enough to explain, for example, the pattern differences between group I and group II strains. Nevertheless, if two strains are to be compared, it is advisable that they be grown on similar media and harvested at the same point in the growth cycle.

**Strain identification.** Besides the use of two-dimensional gels to show similarities between members of certain species, this technique can be used to show differences between similar strains. Note that each of the *R. meliloti* strains (which had patterns very similar to each other) had its own distinct pattern which differed from that of other *R. meliloti* patterns in the relative position of 2 to 20% of the visible protein spots. This can be most easily observed by overlaying one gel over another. Such a pattern is useful for differentiating strains which were presumed to be identical based on serology, antibiotic susceptibility, or phage typing (13).

## DISCUSSION

**Rhizobial taxonomy.** The clearest taxonomic distinction which can be drawn, using gel analysis, is that between the fast- and slow-growing rhizobia. Even in the case of the lotus inoculation group, which contains representatives of each class, the distinction remains valid. It is also interesting that the species whose pattern is most distinctive of its own inoculation group, *R. meliloti*, also shows the least amount of cross-inoculation among group I rhizobia (24). *R. leguminosarum* and *R. trifolii* can be grouped into a single class according to the gel patterns. Again, this grouping matches the observation that these two species readily cross-inoculate their respective hosts, pea and clover (24). It is also not surprising that this is one pair of group I rhizobia between which chromosomal conjugation occurs (8).

Group II rhizobia examined are very similar to each other in terms of overall pattern. By analogy, these strains show a comparable amount of diversity compared with 10 different clinical isolates of *Klebsiella pneumoniae* (G. Roberts, unpublished observations). We argue that this is an indication of similar genetic backgrounds in the group II rhizobia and predict that when a gene transfer system among the group II strains is available, homologous recombination between any two of these strains will occur. For example, deoxyribonucleic acid from lotus rhizobia might be successfully maintained and expressed in *R. japonicum* or other group II strains. This is, of course, in contrast to the situation among the group I strains as a whole.

Figure 2 presents a more detailed arrangement of the strains based on their gel patterns. Strains listed above one another in a column are very similar, whereas any strains whose lines can be traced upwards to a common vertex show at least some distinctive similarities. Several points should be made. (i) In group 1b, some *R. trifolii* strains are more similar to *R. leguminosarum* strains then to other *R. trifolii*. This emphasizes the lack of distinction between these two "species." (ii) In group 1c (diverse group I strains), a number of strains can only be paired with a single, "like" strain, since the diversity among these strains (sesbania rhizobia, *R. phaseoli*, and lotus rhizobia) is so great. (iii) In group, II, *R. lupini* and the lotus rhizobia are closely related to the *R. japonicum* and cowpea rhizobia tested, although a few distinguishing spots do exist for the former inoculation groups (see Fig. 1). (iv) Neither the geographic source nor the species of the parent host explains the groupings in Figs. 2 and 3; for example, *R. trifolii* strains 162Z1, 162S31 and 162P17 have very similar gel patterns, yet are from dissimilar hosts and regions (see Table 1).

It is evident that the current method of taxonomy of *Rhizobium* species based on inoculation properties is inappropriate, especially as the time for genetic analysis of these strains approaches (8, 11, 14). Although protein patterns do not suggest where species lines are to be drawn, they do make predictions on strain relatedness and inoculation group properties. Strains

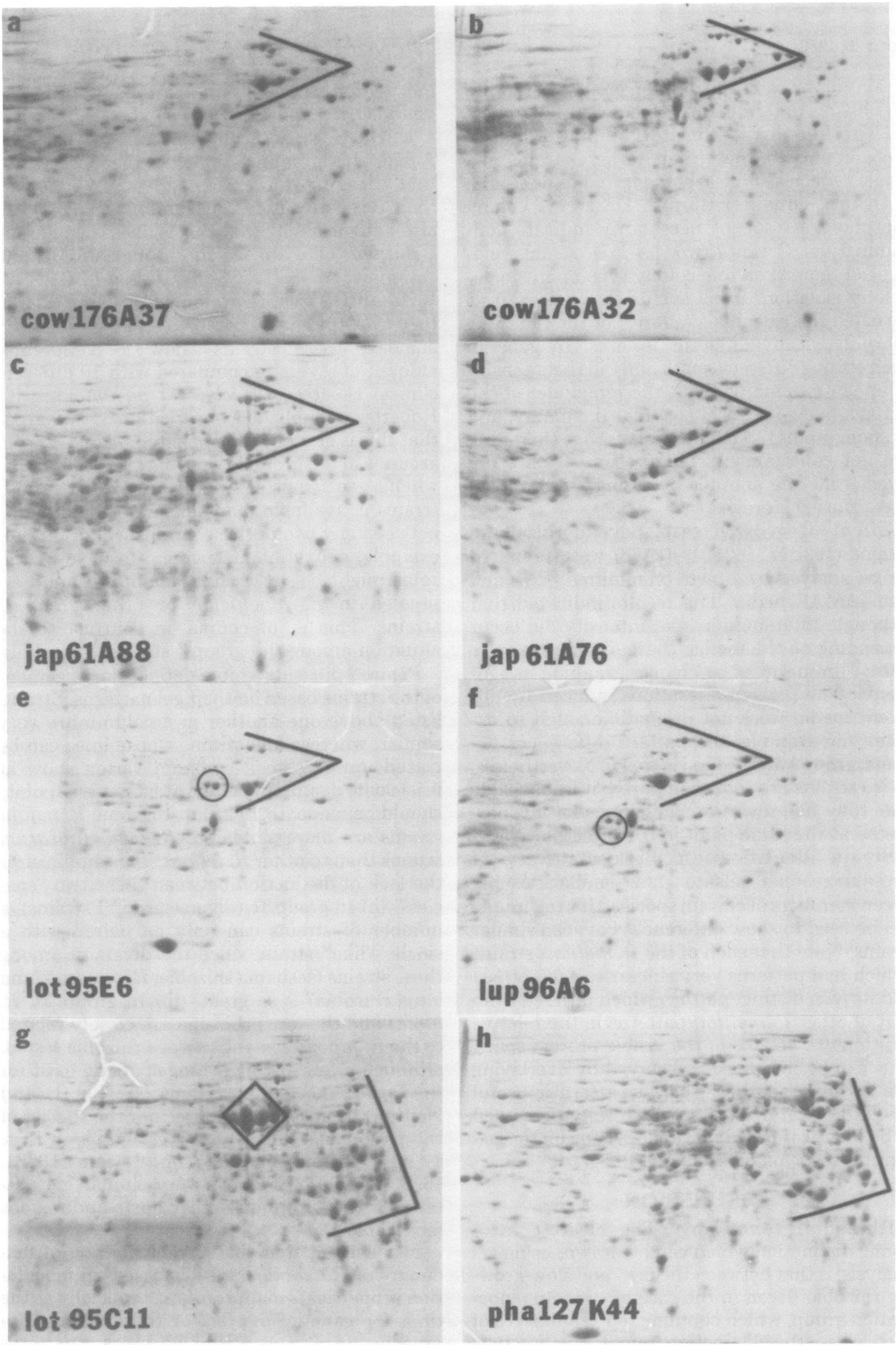
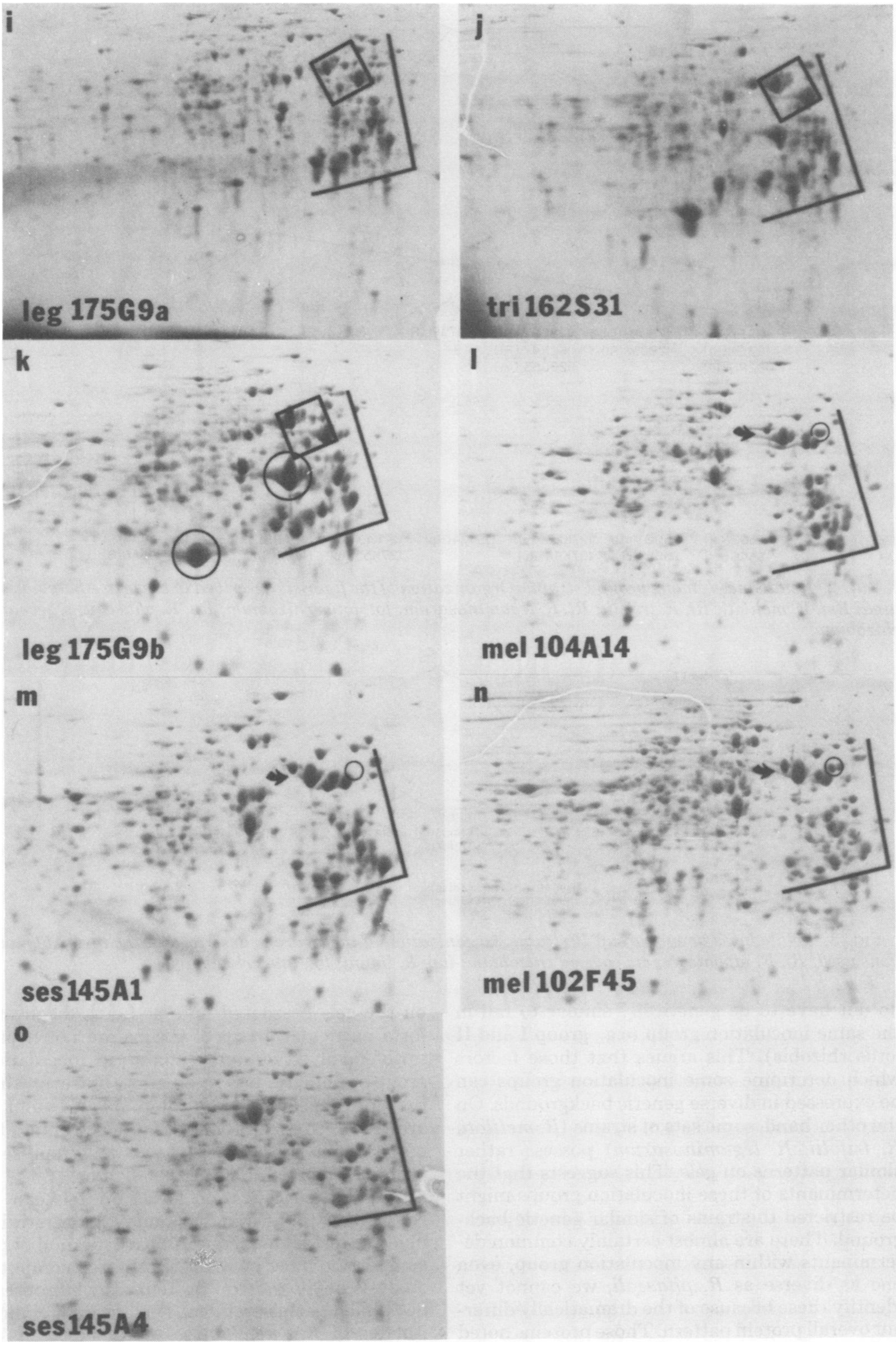



FIG. 1. *Two-dimensional gel patterns of selected Rhizobium strains. In each gel, proteins on the left side are basic (pH 8), and those on the right side are acidic (pH ~4). Those at the top are high molecular weight (>150,000), and those at the bottom are low molecular weight (~15,000). Lines have been placed on the gels to help orient the reader. The patterns marked with circles and squares are discussed in the text. (a–f) Group II rhizobia; (g–o) group I. The following abbreviations are used: cow, cowpea rhizobia; jap, R. japonicum; lot, lotus rhizobia; lup, R. lupini; pha, R. phaseoli; leg, R. leguminosarum; tri, R. trifolii; mel, R. meliloti; ses, sesbania rhizobium.*


i
leg 175G9a
j
tri 162S31
k
leg 175G9b
l
mel 104A14
m
ses 145A1
n
mel 102F45
o
ses 145A4

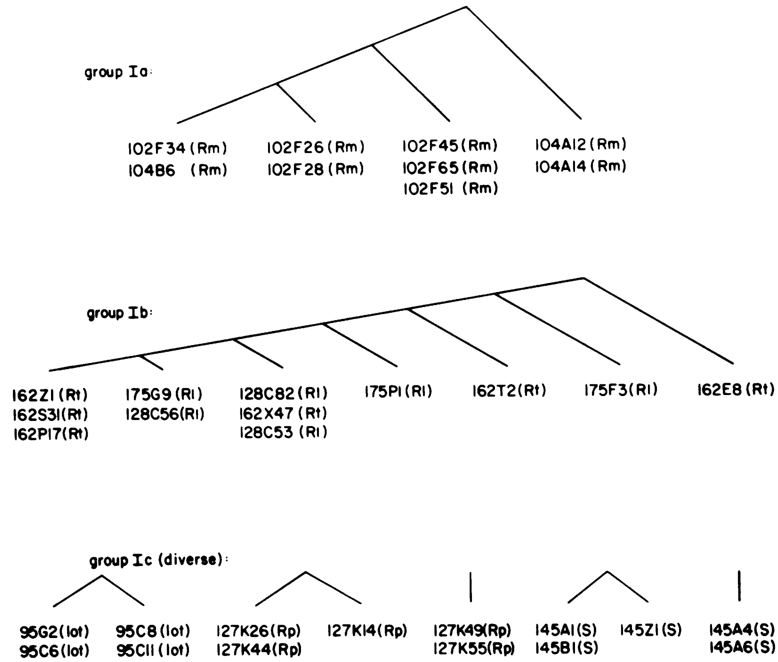



FIG. 2. *Relatedness among group I strains. Organization of the figure is described in the text. Abbreviations used: Rm, R. meliloti; Rt, R. trifolii; Rl, R. leguminosarum; lot, lotus rhizobium; Rp, R. phaseoli; s, sesbania rhizobium.*

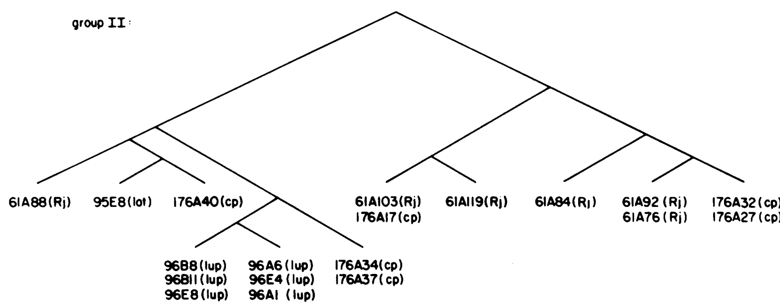



FIG. 3. *Relatedness among group II strains. Organization of the figure is described in the text. Abbreviations used: Rj, R. japonicum; cp, cowpea rhizobium; lup, R. lupini; lot, lotus rhizobium.*

do not have to be genetically similar to fall in the same inoculation group (e.g., group I and II lotus rhizobia). This argues that those factors which determine some inoculation groups can be expressed in diverse genetic backgrounds. On the other hand, some sets of strains (*R. meliloti, R. trifolii, R. leguminosarum*) possess rather similar patterns on gels. This suggests that the determinants of these inoculation groups might be restricted to strains of similar genetic background. There are almost certainly common determinants within any inoculation group, even one as diverse as *R. phaseoli*; we cannot yet identify these because of the dramatically different overall protein pattern. Those proteins noted on the gels presented here as being diagnostic for a particular group of strains are not necessarily involved as determinants in inoculation group specificity. It is interesting, however, that the "lotus-specific" spots noted in Fig. 1e and 1g are similar to each other in their electrophoretic mobility and therefore might actually represent proteins involved in lotus specificity.

Before a classification based on gels can be considered to be reliable, many more strains must be tested and a quantitative method of gel comparison must be made. Perhaps a computer analysis of the general protein distribution can be applied to this problem. Ten different clinical isolates of *K. pneumoniae* (obtained from W.

Silver) were analyzed on two-dimensional gels. These strains were very similar to each other and to *K. pneumoniae* M5a1, a nonclinical isolate, but the patterns were clearly different from that of *Escherichia coli* K-12. Two different *Frankia* isolates from nodulated angiosperms (obtained from J. Torrey) had patterns similar to each other but different from the patterns found in the other bacteria (including actinomycetes) which we have analyzed. Thus, this system may be applied to taxonomy problems of all bacteria and other organisms as well.

When any bacterial system is examined by gels, the most important part of the analysis is the choice of appropriate proteins to serve as markers. The proteins selected should be easily scorable as well as reflective of the other, less visible proteins of the pattern. The position of such benchmark spots would be a reflection of previous evolutionary alterations and therefore taxonomic relatedness.

While gel patterns have been used for taxonomy in other systems, the gel analysis has typically used one-dimensional gels (for example, references 4 and 21). Although these patterns may be useful in some instances, they possess very limited information and tend not to aid in the comparison of rather dissimilar organisms. A problem also exists when the more abundant proteins in the cell do not reflect the overall genotype of the strain. For example, one-dimensional analysis of the extracts used in Fig. 1i would yield rather dissimilar patterns due to the two very abundant proteins circled in Fig. 1k. One of these strains is, however, almost certainly a spontaneous derivative of the other (an event presumably occurring during strain storage), and this extreme relatedness is evidenced by the two-dimensional analysis. A recent report (15) has used two-dimensional gels to identify several strains of spiroplasmas. Although their approach to strain recognition (for identification rather than taxonomy) was similar to our own, their use of radioactive label and gradient gels in the second dimension is very time consuming and unnecessary for the majority of applications.

Another use of this technique is to take advantage of differences in protein patterns between closely related strains. This is useful in cases where one would like to know whether a strain isolated from a complex ecosystem is identical to a strain suspected of being introduced to that ecosystem. In the case of *Rhizobium,* it is important to know whether the strain added to a legume actually is the one found later within the nitrogen-fixing nodules. Many soils have populations of indigenous rhizobia that have not been characterized. If an isolate has a pattern identical (easily tested by overlaying one gel over the other) to that of the inoculated strain, then one can assume that the strains are identical. If only a few spots are different in the two strains, it is possible that mutation has occurred or that plasmid loss or transfer has occurred; but the strains will still clearly have most of their proteins in the same positions and, therefore, one can conclude that the strains are the same.

The technique of two-dimensional gel electrophoresis is used by many laboratories and is easy to master. It is possible to use a colony's worth of cells (taken with a loop) to get enough protein for a gel. One individual in our lab easily runs 60 gels per week, and simple modifications of the apparatus (e.g., larger tanks and plates) could readily increase the rate of gel analysis.

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